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# Original Research Communication

# Regulation of the Activation of Nuclear Factor κB by Mitochondrial Respiratory Function: Evidence for the Reactive Oxygen Species-Dependent and -Independent Pathways

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## ABSTRACT

**Mitochondrial respiratory function regulates the redox status of cells, which, in turn, can control the activation of transcription factors. However, how mitochondria accomplish this modulation is not completely understood. Using the human myelogenous leukemia cells ML-1a, respiration-deficient clone 19 derived from ML-1a, and reconstituted clones, we demonstrated the role of respiratory function in the activation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). Constitutive activation of NF-κB and AP-1 was observed in clone 19, but not in ML-1a, and the constitutive activation observed in clone 19 was completely inhibited in reconstituted clones that have functional mitochondria. Additionally, tumor necrosis factor (TNF)-induced activation of NF-κB and AP-1 observed in ML-1a was greatly reduced in clone 19. These results indicate that mitochondrial respiratory function regulates TNF-induced and constitutive activation of NF-κB and AP-1. We investigated the roles of reactive oxygen species in NF-κB activation. Generation of superoxide detected by hydroethidine, but not hydrogen peroxide detected by dehydrorhodamine 123, was transiently increased by TNF in both of the cells. The antioxidant, pyrrolidine dithiocarbamate, reduced TNF-induced, but not the constitutive, NF-κB activation. These results indicate that the increase in superoxide generation might be involved in TNF-induced, but not in constitutive, NF-κB activation. Our results thus demonstrate the involvement of mitochondrial respiratory function in the activation of reactive oxygen species-dependent and -independent pathways for NF-κB activation.** *Antioxid. Redox Signal.* 4, 945–955.

## INTRODUCTION

MITOCHONDRIA are considered a key organelle for generating cellular energy and have recently been considered critical for apoptosis signaling (22). Because it has been estimated that ~2% of the rate of state 4 respiration by mitochondria results in production of reactive oxygen species (ROS) (6), mitochondrial respiratory chains (MRC) have been considered the major source of ROS. Electron transfer in MRC principally reduces oxygen molecules to water and generates membrane potential to make ATP. At the same time, all of the reduced flavins, non-heme iron proteins, quinols, and especially semiquinones in MRC can donate electrons to the oxygen molecule to generate ROS. Several factors, such as oxygen concentration, coupling/uncoupling, the state of respiration, the concentration of respiratory substrate, activities of transporter of ADP, respiration substrate, and antioxidants, can control respiratory function and ROS generation from MRC (39). Inhibition of certain MRC can increase the generation of ROS (6, 42), indicating that changing the electron transfer in MRC can regulate ROS generation. Thus, the regulation of mitochondrial respiratory function can affect

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redox responses and ROS generation; however, how mitochondria regulate these changes in cells has not been clearly elucidated.

Human mitochondrial DNA (mtDNA) has a remarkably small size of 16,569 bp, compared with nuclear DNA of $\sim 10^9$, and encodes only some part of proteins in the MRC: 13 polypeptides [seven polypeptides in complex I (NADH-ubiquinone oxidoreductase), cytochrome *b* in complex III (ubiquinol-cytochrome *c* oxidoreductase), three polypeptides in complex IV (ferrocytochrome *c*-oxygen oxidoreductase), and two polypeptides in complex V (Fo-F1 ATPase)], 22 transfer RNA, and two ribosomal RNA. Several mtDNA mutations have been considered as the cause of diseases such as the Kearns–Sayre syndrome (34), myoclonus epilepsy with ragged red fibers syndrome (38), and mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes syndrome (15). Recent improvement of the techniques to detect mutation revealed the increase in somatic mtDNA mutation in various tumors and tumor cell lines (9, 47) and myocardial ischemia (43). Mitochondrial mutation increases with age and can be considered one of the causes of aging. Principally, these mutations could contribute to these diseases; however, the mechanisms involved have not been characterized. To examine the cause of these diseases, it is critically important to investigate the roles of mitochondrial respiratory functions, which are affected by mtDNA changes, on the signal transduction pathways.

One potential mediator, nuclear factor κB (NF-κB), is a ubiquitous transcription factor that is activated by several stimuli, such as cytokines, oxidative stress, and hypoxic conditions. The regulation of the most common form of NF-κB involves the interaction of at least three distinct subunits, p50, p65, and IκBα. In the cytoplasm, the major form of NF-κB is found in its inactive state with the inhibitory subunit, IκBα, bound to the p50–p65 heterodimer (4). Upon activation, IκBα is phosphorylated and degraded, and then the p50–p65 heterodimer complex is translocated from the cytosol to the nucleus where it binds to the κB motif (2). It was demonstrated that the NF-κB activation can be mediated through the generation of ROS, because NF-κB can be inhibited by antioxidant, and the generation of ROS can induce NF-κB (35). However, the exact mechanisms underlying its action have not been fully investigated. Additionally, it was also indicated that the requirement of ROS for NF-κB activation is dependent on the stimulus and the cell type (5, 7, 35).

As ROS can be generated from MRC and the role of ROS in NF-κB activation is also known, the role of mitochondrial ROS generation in NF-κB activation has been studied. Antimycin A, an inhibitor of MRC complex III, can generate ROS (6) and activate NF-κB (13). Furthermore, inhibitors of MRC complex I rotenone inhibit tumor necrosis factor (TNF)-induced and hydrogen peroxide-induced NF-κB activation (19). These results indicate that the change in ROS generation and/or redox state in MRC can regulate NF-κB activation.

Previously, we established cell lines lacking mtDNA and thus lacking mitochondrial respiratory function (17). This set of cells provides the opportunity to investigate the role of mitochondrial respiratory function in biological responses. We showed that TNF and serum starvation could not induce apoptosis in respiration-deficient cells, whereas they did induce apoptosis in parental cells and reconstituted cells (17), indicating the role of mitochondrial respiratory function in apoptosis induced by TNF and serum starvation. Additionally, the regulation of nuclear gene expression has been investigated as retrograde regulation (33).

To investigate the roles of mitochondrial respiratory function in activation of transcription factors such as NF-κB and activator protein-1 (AP-1), we used a respiration-deficient clone and reconstituted cybrids. We showed that mitochondrial respiratory activity highly correlates with the ability of TNF to activate NF-κB. In addition, constitutive NF-κB activation occurs in a respiration-deficient clone, but is inhibited in the cells where mtDNA and respiratory function are reconstituted. Our study indicates that the mitochondria are the key organelle for NF-κB activation. We also showed the roles of mitochondrial respiratory function in ROS-dependent and -independent NF-κB activation.

## MATERIALS AND METHODS

### *Reagents*

RPMI 1640 medium, gentamicin, and fetal calf serum (FCS) were obtained from GIBCO (Grand Island, NY, U.S.A.). Phenylmethanesulfonyl fluoride (PMSF), aprotinin, leupeptin, pepstatin, glycerol, dithiothreitol (DTT), EGTA, EDTA, uridine, glucose, pyruvate, Triton X-100, pyrrolidine dithiocarbamate (PDTC), dimethyl sulfoxide, sucrose, HEPES, and human TNF were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hydroethidine (HE) and dihydrorhodamine 123 (DHR123) were purchased from Molecular Probes Inc. (Eugene, OR, U.S.A.). 2-Methoxyestradiol was a kind gift from Dr. Peng Huang.

### *Cell culture*

Myelogenous leukemia ML-1a cells were obtained from Dr. Ken Takeda (Showa University, Tokyo, Japan). ML-1a and reconstituted clones (P1, P2, and P3) were grown in RPMI 1640 medium supplemented with 10% FCS and gentamicin (50 μg/ml) (essential medium). Clone 19 was grown in RPMI 1640 medium with 10% FCS and gentamicin (50 μg/ml) supplemented with 4.5 mg/ml glucose, 50 μg/ml uridine, and 100 μg/ml pyruvate (enriched medium). The cells were seeded at a density of $1 \times 10^5$ cells/ml in T-25 flasks (Falcon 3013, Becton–Dickinson Labware, Lincoln Park, NJ, U.S.A.) containing 10 ml of medium and grown at 37°C in an atmosphere of 95% air and 5% $CO_2$. Cell cultures were split every 3–5 days.

### *NF-κB assays*

ML-1a cells ($2 \times 10^6$ cells/ml) were treated with the indicated amounts of TNF in the presence of the indicated amounts of inhibitors for the indicated times at 37°C. Nu-

clear extracts were prepared according to Schreiber *et al.* (36). In brief, $2 \times 10^6$ cells were washed with cold phosphate-buffered saline (PBS) and suspended in 0.4 ml of lysis buffer (10 m*M* HEPES, pH 7.9, 10 m*M* KCl, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 1 m*M* DTT, 0.5 m*M* PMSF, 2.0 μg/ml leupeptin, 2.0 μg/ml aprotinin, and 0.5 μg/ml benzamidine). The cells were allowed to swell on ice for 15 min, after which 25 μl of 10% NP-40 was added. The tube was then vigorously shaken on a vortex mixer for 10 s, and homogenate then centrifuged for 30 s in a microfuge. The nuclear pellet was resuspended in 25 μl of ice-cold nuclear extraction buffer (20 m*M* HEPES, pH 7.9, 0.4 *M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* DTT, 1 m*M* PMSF, 2.0 μg/ml leupeptin, 2.0 mg/ml aprotinin, and 0.5 μg/ml benzamidine), and the tube was incubated on ice for 30 min with intermittent mixing. This nuclear extract was then centrifuged for 5 min in a microfuge at 4°C, and the supernatant was frozen at −70°C. The protein content was measured by the method of Bradford (8).

Electrophoretic mobility shift assays (EMSA) were performed by incubating 4–5 mg of nuclear extract with 12 fmol of $^{32}$P-end-labeled 45mer double-stranded NF-κB oligonucleotide from human immunodeficiency virus-1 long terminal repeat (5′−TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGG−3′) (30) in the presence of 0.5 mg of poly(dI-dC) in a binding buffer [25 m*M* HEPES, pH 7.9, 0.5 m*M* EDTA, 0.5 m*M* DTT, 1% NP-40, 5% glycerol, and 50 m*M* NaCl (16, 30)] for 20 min at 37°C. The DNA–protein complex formed was separated from free oligonucleotide on a 6.6% native polyacrylamide gel using buffer containing 50 m*M* Tris/200 m*M* glycine, pH 8.5, and 1 m*M* EDTA (16). The gel was fixed in 10% acetic acid and dried. Quantitation and visualization of radioactive bands were carried out by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.) using ImageQuant software.

### *AP-1 assays*

The activation of AP-1 was determined as described (25).

### *Detection of hydrogen peroxide by DHR123*

The detection of intracellular hydrogen peroxide by DHR123 was determined by flow cytometry as described (46). In brief, cells ($1 \times 10^6$) were incubated with 10 μ*M* DHR123 for 30 min at 37°C, washed with PBS, and resuspended in 0.5 ml of PBS. Rhodamine 123 fluorescence intensity resulting from DHR123 oxidation was measured by FACScan flow cytometry (Becton–Dickinson, Mountain View, CA, U.S.A.) with excitation at 488 nm and was detected at 530 nm.

### *Detection of superoxide by HE*

The detection of intracellular superoxide by HE was determined by flow cytometry by modified methods described

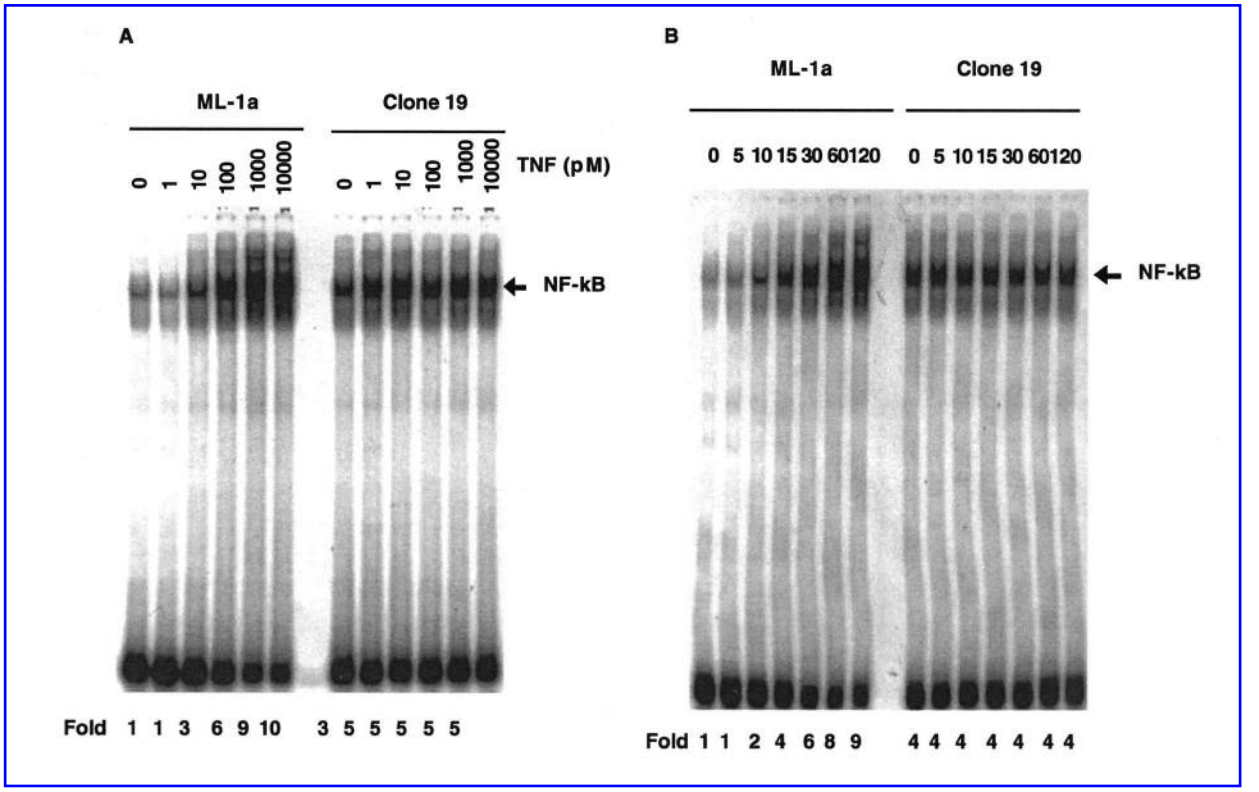


**FIG. 1. Activation of NF-κB by TNF in ML-1a and clone 19.** Nuclear extracts were prepared from $2 \times 10^6$ ML-1a and clone 19 cells incubated with or without 0.1 n*M* (**B**) or the indicated amount of TNF (**A**) for 1 h (A) or the indicated time (B), and NF-κB activation was assayed as described under Materials and Methods.

(18). In brief, cells ($1 \times 10^6$) were incubated with 10 $\mu M$ HE for 30 min at 37°C, washed with PBS, and resuspended in 0.5 ml of PBS. HE fluorescence intensity resulting from HE oxidation was measured by FACScan flow cytometry with excitation at 488 nm and was detected at 630 nm.

## RESULTS

### *Constitutives activation of NF-κB and reduction of TNF-induced NF-κB activation in respiratory-deficient clone*

We had isolated mtDNA- and respiration-deficient clone 19 from human myelogenous leukemia ML-1a by continuous exposure to low concentrations of ethidium bromide (17). We confirmed that clone 19 is respiration-deficient and mtDNA-deficient by the following results: (a) We could not detect mtDNA by PCR in clone 19 (data not shown). (b) State 3 respiration using digitonized cells with succinate as substrate in clone 19 was <1% of ML-1a (17). (c) Clone 19 could not survive in the absence of pyruvate or uridine, which is characteristic of respiration- and mtDNA-deficient cells (20). To investigate the role of mitochondrial respiratory function in TNF-induced signaling, we used ML-1a and clone 19 and compared the signal for NF-κB activation. Figure 1A shows the dose-dependent activation of NF-κB by TNF in ML-1a cells and clone 19 cells. Up to 10-fold activation of NF-κB by TNF was observed in ML-1a. In clone 19, constitutive activation of NF-κB was observed (threefold compared with ML-1a), but NF-κB activation induced by TNF was much lower (10-fold vs. 1.7-fold). Figure 1B shows the time-dependent activation of NF-κB by TNF in ML-1a, and in clone 19. We could detect TNF-induced NF-κB activation in ML-1a, but not in clone 19. We could not detect a significant change of the amount of TNF receptor in clone 19 estimated by binding assay (data not shown), and TNF-induced apoptosis, but not differentiation, was inhibited in clone 19 (17). These results indicate that specific, but not all, signals for TNF were inhibited in clone 19.

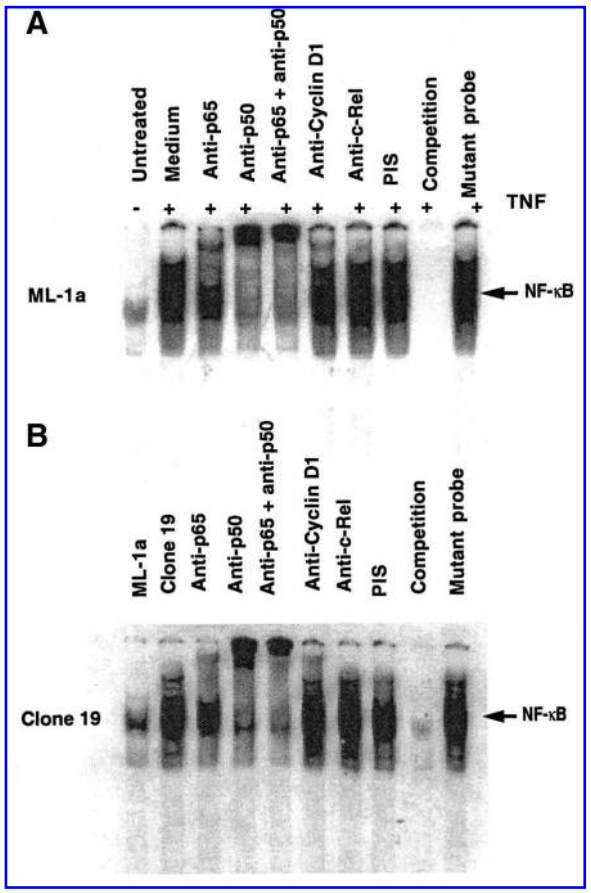


**FIG. 2. Supershift and specificity of NF-κB.** Nuclear extracts were prepared from $2 \times 10^6$ ML-1a cells incubated with 0.1 n$M$ TNF for 1 h (**A**), and $2 \times 10^6$ clone 19 cells (**B**) were incubated with different antibodies, unlabeled oligo, or mutated oligo and then assayed for NF-κB as described under Materials and Methods.

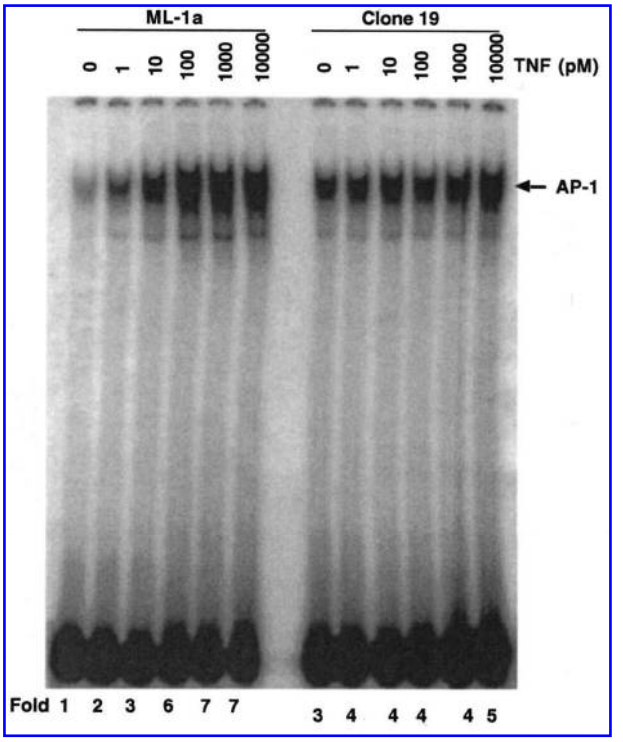


**FIG. 3. Activation of AP-1 by TNF in ML-1a and clone 19.** Nuclear extracts were prepared from $2 \times 10^6$ ML-1a and clone 19 cells incubated with the indicated amount of TNF for 1 h, and AP-1 activation was assayed as described under Materials and Methods.

More importantly, constitutive activation of NF-κB in clone 19 was observed. TNF-induced NF-κB activation in clone 19 was not observed (Fig. 1A) or significantly weaker (Fig. 1B) than that in ML-1a in five independent experiments. These results indicate that respiratory function is involved in TNF-induced NF-κB activation and that NF-κB is constitutively activated in the absence of respiratory function.

Principally, NF-κB exists in its inactive state in the cytoplasm as a complex consisting of p50, p65, and IκBα. When

activated, the p60–p55 complex is dissociated from IκBα. To examine the composition of spontaneously activated NF-κB in clone 19, we incubated nuclear extracts with antibodies to p50 and p65 and then analyzed the extracts by EMSA. As a positive control, we used TNF-treated ML-1a cells, where p50 and p65 were transported to the nucleus. Anti-p50 nearly completely shifted all the NF-κB bands from TNF-treated ML-1a and clone 19 and anti-p65 shifted some of the of NF-κB bands from both (Fig. 2). This supershift was specific, as antibodies to c-Rel, cyclin D1, or preimmune serum (PIS) had no effect. Specificity was also indicated by the disappearance of this band when unlabeled probe was used and its persistence when the oligonucleotide with mutated NF-κB site was used (Fig. 2). These results indicate that both p50 and p65 were transported to the nucleus in clone 19 and TNF-treated ML-1a.

### *Roles of mitochondrial respiratory function in AP-1 activation*

We next treated ML-1a and clone 19 with TNF and measured the activation of AP-1. The activation of AP-1 by TNF was dose-dependent in ML-1a (Fig. 3). Constitutive activation of AP-1 occurred in clone 19 cells (threefold compared with ML-1a), but the TNF-induced activation of AP-1 was lower in clone 19 than in ML-1a (sevenfold vs. 1.7-fold). These results indicate that, in the absence of respiratory function, AP-1 was constitutively activated and that the activation of AP-1 by TNF was much lower.

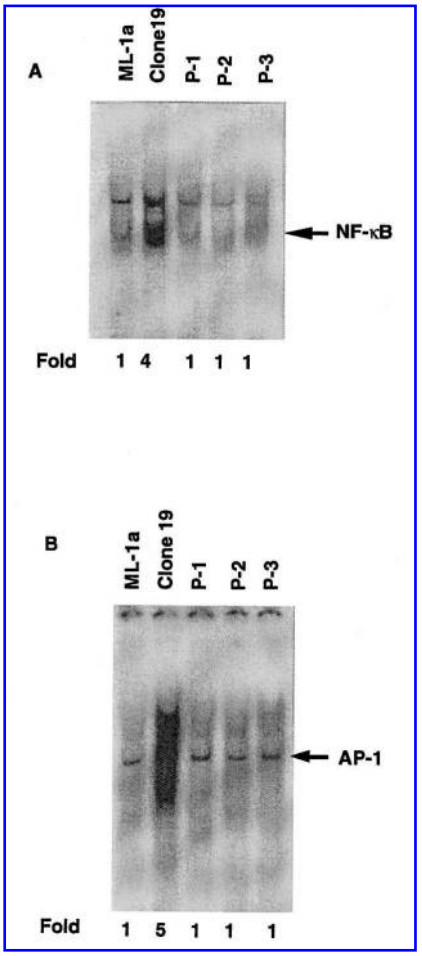


**FIG. 4. NF-κB (A) and AP-1 (B) in ML-1a, respiration-deficient clone 19, and reconstituted clones P1, P2, and P3.** Nuclear extracts were prepared from 2 × $10^6$ ML-1a, clone 19, P1, P2, and P3 cells, and NF-κB (**A**) and AP-1 (**B**) were assayed as described under Materials and Methods.

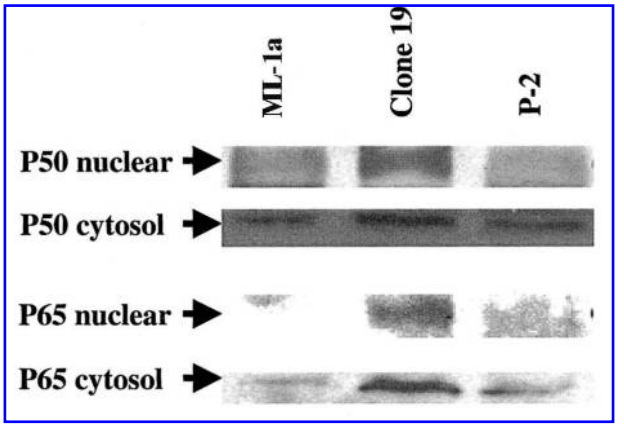


**FIG. 5. Constitutive NF-κB activation in clone 19 determined by western blotting.** Nuclear and cytosolic extracts from 1 × $10^7$ ML-1a, clone 19, and P2 cells were applied to western blotting using anti-p50 and p65 forms of NF-κB antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).

### *Inhibition of constitutive activation of NF-κB and AP-1 by the reconstitution of mtDNA and respiratory function*

To confirm that the effect observed in clone 19 was due to the defect in respiratory function caused by the depletion of mtDNA induced by ethidium bromide treatment, mtDNA and mitochondrial respiratory function were reconstituted by the fusion of clone 19 with platelets, which have normal mtDNA without nuclear DNA. We obtained cybrid clones (P1, P2, and P3) that had respiratory function (17). State 3 respiration of ML-1a, clone 19, P1, P2, and P3 was 6.7, 0.07, 11.0, 4.0, and 4.0, respectively, indicating that clone 19 lost respiratory activity and P1, P2, and P3 recovered respiratory activity (17). As shown in Fig. 4A, the constitutive NF-κB activation, a characteristic of clone 19, was greatly inhibited in reconstituted clones P1, P2, and P3, indicating the regulation of constitutive NF-κB activation by mitochondrial respiratory function. Constitutive activation of AP-1 in clone 19 was also greatly inhibited in clones P1, P2, and P3 (Fig. 4B). Therefore, respiratory function is inhibiting the constitutive activation of NF-κB and AP-1 in the cells with intact mitochondria (ML-1a, P1, P2, and P3).

We further investigated constitutive activation of NF-κB by western blotting using anti-p50 and anti-p65. The protein amount of the nuclear fraction of ML-1a, clone 19, and P2 was 91, 101, and 93 μg/1 × $10^7$ cells, respectively, and that of the cytosolic fraction of ML-1a, clone 19, and P2 was 559, 650, and 877 μg/1 × $10^7$ cells, respectively, indicating that the total protein amount in these cell lines was approximately the same. We detected a greater amount of the p50 and p65

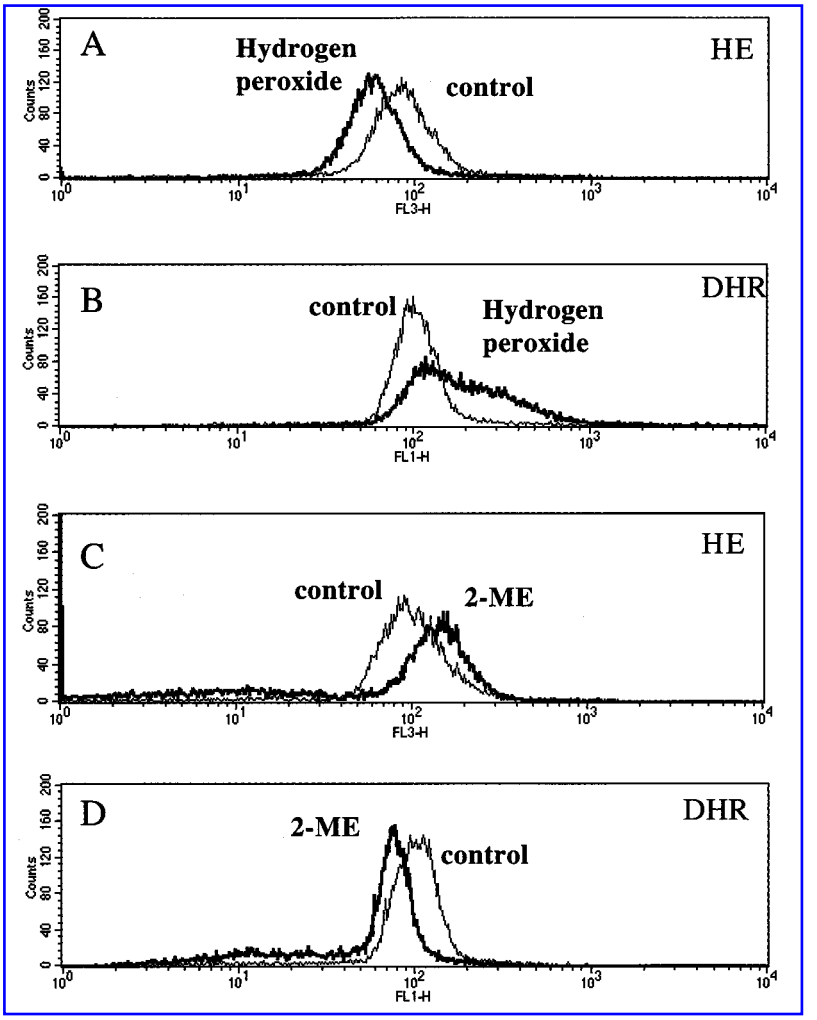


**FIG. 6. The effect of hydrogen peroxide and 2-methoxyestradiol on the fluorescence in ML-1a cells treated with HE and DHR123.** ML-1a cells were incubated with or without 10 μ*M* hydrogen peroxide (**A** and **B**) or 1 m*M* 2-methoxyestradiol (2-ME) (**C** and **D**) for 7.5 h and then further incubated in the presence of 10 μ*M* HE (A and C) or 10 μ*M* DHR123 (B and D) for 30 min. Then fluorescence of each cell line was detected by FACS analysis as described under Materials and Methods.

forms of NF-κB in the cytosolic and nuclear fractions of clone 19 than in ML-1a or P2 (Fig. 5).

### *Involvement of superoxide in TNF-induced NF-κB, but not in constitutive NF-κB activation*

It was demonstrated that the NF-κB activation can be mediated through the generation of ROS, so we investigated whether one of the pathways for NF-κB activation (TNF-induced NF-κB activation and constitutive activation of NF-κB) requires ROS generation. First, we investigated the effect of TNF on ROS generation in ML-1a and clone 19. An electron from MRC is donated to oxygen molecules to generate superoxide, and superoxide is dismutated to hydrogen peroxide by superoxide dismutase, and we decided to investigate the generation of hydrogen peroxide and superoxide. We used DHR123, which is considered to detect intracellular hydrogen peroxide (46), and this method is widely used to detect ROS generation. We also used the fluorescent dye HE, which is considered to detect superoxide (18, 24).

To confirm that HE detects superoxide and DHR123 detects hydrogen peroxide, we used 2-methoxyestradiol as a superoxide generator (18) and hydrogen peroxide. As shown in Fig. 6A and B, hydrogen peroxide enhanced the intensity of fluorescence in DHR123-treated, but not HE-treated, ML-1a, indicating that DHR123, but not HE, could detect hydrogen peroxide. In contrast, 2-methoxyestradiol enhanced the intensity of fluorescence in HE- but not DHR123-treated ML-1a (Fig. 6C and D), indicating that HE, but not DHR123, could detect superoxide generation.

ML-1a and clone 19 were incubated with TNF in the presence of DHR123 and tested for their effect on the generation of hydrogen peroxide. We could not detect any significant increase in hydrogen peroxide generation in ML-1a and clone 19 by TNF (Fig. 7A and B). We could detect a slight decrease in hydrogen peroxide generation in clone 19 compared with ML-1a. ML-1a and clone 19 were incubated with TNF in the presence of HE and tested for their effect on the generation of superoxide. We could detect a slight, but significant, increase in superoxide generation by TNF in ML-1a and clone 19 (Fig. 7C

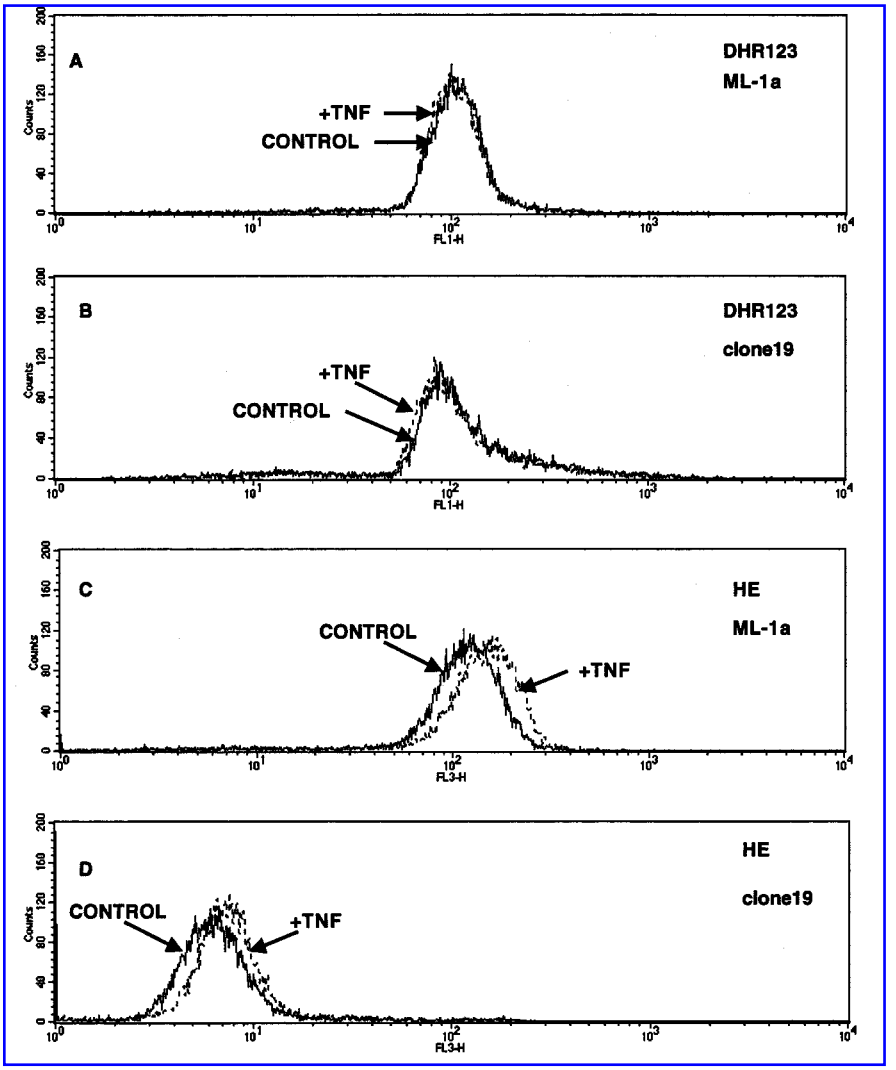


**FIG. 7. Generation of superoxide and hydrogen peroxide in ML-1a and clone 19 and the effect of TNF.** ML-1a (**A** and **C**) and clone 19 (**B** and **D**) were incubated with 1 n*M* TNF in the presence of 10 μ*M* DHR123 (A and B) or 10 μ*M* HE (C and D) for 30 min. Then superoxide and hydrogen peroxide generation was assayed as described under Materials and Methods.

and D). Interestingly, the intensity of fluorescence in the HE-treated clone 19 was extremely lower than that in HE-treated ML-1a (Fig. 7C and D). This might be caused by the decrease in superoxide generation in clone 19 or the reduction of the binding activity of ethidium, which is an oxidative product of HE. We investigated whether depletion of mtDNA reduced the binding of ethidium to DNA in clone 19. We incubated ML-1a and clone 19 with 0.2, 2, and 20 μ*M* ethidium bromide, and determined the binding of ethidium to both of the cell lines by FACS analysis. The binding of ethidium to ML-1a and clone 19 was approximately the same, but HE-treated clone 19 showed a greatly reduced intensity of fluorescence compared with HE-treated ML-1a (Fig. 8). This result indicates that superoxide generation is greatly reduced in clone 19. Although the ratio of the increase in superoxide generation by TNF in clone 19 was approximately the same as that in ML-1a, the net amount of the increase in superoxide generation by TNF in clone 19 was much smaller than that in ML-1a.

Next, we used the antioxidant, PDTC, and investigated the role of ROS in NF-κB activation by TNF in ML-1a and in constitutive NF-κB activation in clone 19. As we expected, PDTC inhibited TNF-induced NF-κB activation in a dose-dependent fashion (Fig. 9A), but did not inhibit constitutive NF-κB activation in clone 19 (Fig. 9B). These results suggest that PDTC inhibited TNF-induced superoxide generation leading to NF-κB activation, but not constitutive NF-κB activation.

## DISCUSSION

In this report, we investigated the role of mitochondrial respiratory function in the activation and regulation of the transcription factors NF-κB and AP-1. We used a clone that has deficiencies in mitochondrial respiration as a result of the depletion of mtDNA by ethidium bromide treatment, and sev-

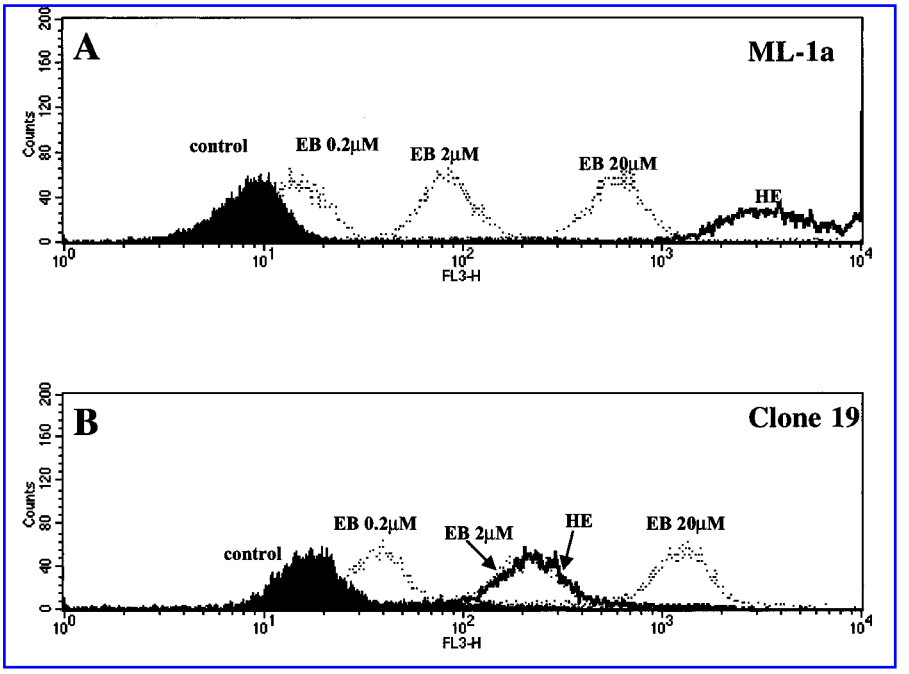


**FIG. 8. Binding of ethidium to ML-1a and clone 19.** ML-1a and clone 19 cells were incubated with or without the indicated amount of ethidium bromide (EB) or 10 μ*M* HE for 30 min. Then the fluorescence of each cell line was detected by FACS analysis as described under Materials and Methods.

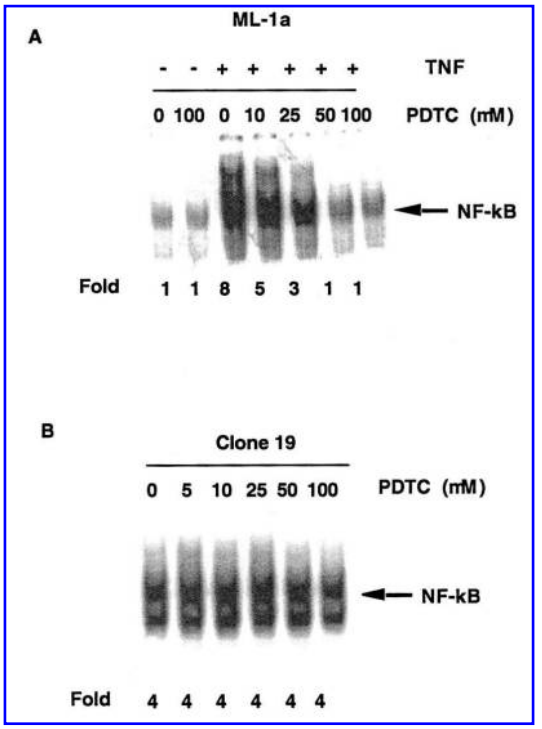


**FIG. 9. The effect of antioxidant PDTC on TNF-induced NF-κB in ML-1a and constitutive NF-κB in clone 19.** ML-1a cells ($2 \times 10^6$) were incubated with or without 0.1 n*M* TNF in the presence of the indicated amounts of PDTC (**A**). Clone 19 cells ($2 \times 10^6$) were incubated in the presence of the indicated amounts of PDTC (**B**). After treatment, nuclear extracts were prepared, and NF-κB activation was assayed as described under Materials and Methods.

eral reconstituted clones derived from respiration-deficient cells fused with platelets. Our results clearly demonstrate that the transcription factors NF-κB and AP-1 were constitutively activated in respiration-deficient clones, and such activation was inhibited in reconstituted clones by the transfer of mtDNA from platelets. We also showed that ROS are involved in TNF-induced NF-κB activation, but not in constitutive activation of NF-κB in clone 19. Inhibition of TNF-induced NF-κB activation in clone 19 was not caused by the reduction of TNF receptor for the following reasons: (a) Binding of labeled TNF to clone 19 was approximately the same as that to ML-1a (data not shown); and (b) TNF-induced differentiation, but not NF-κB activation nor apoptosis induction, was not inhibited (17).

The interactions between nuclear and mitochondrial genomes have been studied (33). Communication from mitochondria to nuclei has been studied principally using mtDNA-deficient yeast cells (3, 32). In mammalian system, Schulze-Osthoff *et al.* first described that mtDNA-less cells showed reduction of TNF-induced NF-κB activation possibly through the change in ROS generation from mitochondria (37). Our observation is closely related, but slightly different from their observations. They demonstrated that NF-κB activation by TNF in mtDNA-less cells is greatly reduced, as we also observed in clone 19. However, their mtDNA-less cells did not express constitutive NF-κB activation. This discrepancy might reside in the differences in the cell lines (mouse fibrosarcoma L929 cells versus human myelogenous leukemia ML-1a) or the differences in the remaining respiratory function in the clones. In the latter case, clone 19 has significantly lower respiratory activity than their "mtDNA-less" cells.

Miranda *et al.* reported that (a) NF-κB is constitutively activated in mtDNA-deficient cells, (b) hydrogen peroxide generation was enhanced in mtDNA-deficient cells, and (c) NF-κB activation can be inhibited by PDTC (28). From these results, they concluded that constitutive NF-κB in mtDNA-deficient cells was activated through ROS-dependent mechanisms (28). Most of the possible sites in MRC for ROS generation in mtDNA-deficient cells could not generate ROS, because only complex II is intact in mtDNA-deficient cells. Especially, coenzyme Q, which is considered a key generator of ROS, would be more oxidized because seven proteins in complex I are missing in clone 19 and would thus generate an oxidized state downstream in the electron transport chains (complex III and IV). Therefore, superoxide generation from MRC might be reduced in mtDNA-deficient clone 19 (Fig. 7). Alternatively, as intracellular oxygen concentration is increased by the lack of respiration in respiration-deficient clone 19, ROS generation from sites other than MRC might increase. That might be one of the possible reasons why the intracellular concentrations of hydrogen peroxide in ML-1a and clone 19 were comparable even though superoxide levels differed. That may also explain the increase in hydrogen peroxide in mtDNA-deficient cells obtained by Miranda *et al.* (28), possibly because their cells might generate more hydrogen peroxide from the site other than mitochondria. Therefore, the discrepancy between our results and the results from Dr. Miranda might arise from the source of ROS and/or the species of ROS. As the antioxidant PDTC inhibited TNF-induced NF-κB activation, but not constitutive NF-κB activation, in clone 19, constitutive NF-κB activation must be independent of ROS at least in clone 19. The possible explanation for the discrepancy in the sensitivity to antioxidant (PDTC) might arise from the difference in the cell type. However, we also detected PDTC-insensitive NF-κB activation in mtDNA-deficient B143 cells (data not shown).

We could detect TNF-induced increase in superoxide, but not hydrogen peroxide, in ML-1a. As antioxidant PDTC reduced TNF-induced NF-κB activation, superoxide generation by TNF might be critically important for TNF-induced NF-κB activation. Although the ratio of the increase in superoxide generation by TNF in clone 19 was approximately the same as that in ML-1a, the net amount of the increase in superoxide generation by TNF in clone 19 was much smaller than that in ML-1a. The fact that the amount of TNF-induced superoxide generation in clone 19 was smaller than that in ML-1a also can explain the reduction of NF-κB activation in clone 19. We are not sure why we could not detect the TNF-induced increase in hydrogen peroxide. One possible explanation is that the intracellular concentration of hydrogen peroxide is greatly higher than that of superoxide, and therefore, an increase in hydrogen peroxide followed by an increase in superoxide is beyond the limit of detection. Additionally, the level of hydrogen peroxide might be well regulated to a certain level by mitochondria or other organelle (12, 23, 27, 29). Another possible explanation for the reduction of TNF-induced NF-κB is that constitutive NF-κB activation may increase the synthesis of IκBα and thus inhibit the TNF-induced NF-κB activation (40).

Original studies of the constitutive activation of NF-κB were reported in B cells (26), T cells (21), and monocytes (10). One of the possible mechanisms of constitutive NF-κB activation was characterized in Hodgkin/Reed Sternberg cells, in which abnormal expression of IκBα, which is supposed to inhibit NF-κB activation, was observed (45). Emmerich *et al.* showed that the possible cause of the constitutive activation of NF-κB is the mutation in the IκBα gene (11), resulting in C-terminally truncated proteins, which are presumably not able to inhibit NF-κB activation; however, they could not find the mutation in all of the cell lines that show constitutive NF-κB activation. Constitutive NF-κB activation in clone 19, however, was inhibited by the transfer of mtDNA from a healthy donor, ruling out the mutation of IκBα as a cause. Autocrine generation of TNF and in turn the induction of NF-κB also could be the cause of constitutive NF-κB activation (14), but that possibility was also ruled out because PDTC could inhibit NF-κB activation by autocrine generation of TNF, but could not inhibit constitutive NF-κB activation in clone 19.

Another cause of constitutive activation of NF-κB is the hypophosphorylation of IκBβ, which acts as chaperone and protects NF-κB from being bound and inhibited by IκBα and drawn into the nucleus (31, 41). Additionally, bcl-3, a homologue of the IκB proteins, has been shown to react with the cytoplasmic pool of p50 complexed with its precursor, p105, thereby forming p50 homodimers that translocate to the nucleus in a p50/p50/Bcl-3 complex (44). We detected the increase in p50 and p65 form of NF-κB in the nuclear fraction and in the cytosolic fraction in clone 19, indicating that total p50 and p65 form of NF-κB was up-regulated by the depletion of mtDNA. Recently, Arnould *et al.* (1) demonstrated that depletion of mtDNA enhanced the intracellular $Ca^{2+}$ concentration that leads to the activation of cyclic AMP-responsive element-binding protein. It is likely that a change other than the increase in ROS, such as the increase in $Ca^{2+}$ concentration caused by the depletion of mtDNA, might lead to constitutive NF-κB activation, but further investigation is necessary to establish the detailed mechanisms involved in constitutive activation of NF-κB in respiration-deficient cells.

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## ABBREVIATIONS

AP-1, activator protein-1; DHR123, dihydrorhodamine 123; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assays; FCS, fetal calf serum; HE, hydroxyethidine; MRC, mitochondrial respiratory chain; mtDNA, mitochondrial DNA; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; PDTC, pyrrolidine dithiocarbamate; PIS, preimmune serum; PMSF, phenylmethanesulfonyl fluoride; ROS, reactive oxygen species; TNF, tumor necrosis factor.

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